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(54) Title: ANTIBODIES SPECIFIC FOR D-MYO-INOSITOL 1,4,5-TRISPHOSPHATE

(57) Abstract

Antibodies against Ins(1,4,5)P_3, were raised by immunizing rabbits with two types of IP_3-BSA conjugates which were synthesized by covalently coupling Ins(1,4,5)P_3 to the carrier protein via alkyl linkages. The anti-Ins(1,4,5)P_3 antibodies were detected by an ELISA where Ins(1,4,5)P_3 was covalently attached to a microplate well surface. Both antiserum preparations showed specific binding with Ins(1,4,5)P_3. The specificity of these antibodies was enhanced by affinity purification for the antiserum through Ins(1,4,5)P_3-agarose chromatography. The affinity-purified antibodies have IC_{50} values of 12 nM and 730 nM for Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4, respectively. These antibodies showed many properties similar to those of biologically relevant receptors for Ins(1,4,5)P_3.

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ANTIBODIES SPECIFIC FOR D-MYO-INOSITOL 1,4,5-TRISPHOSPHATE

Background and Brief Summary of the Invention

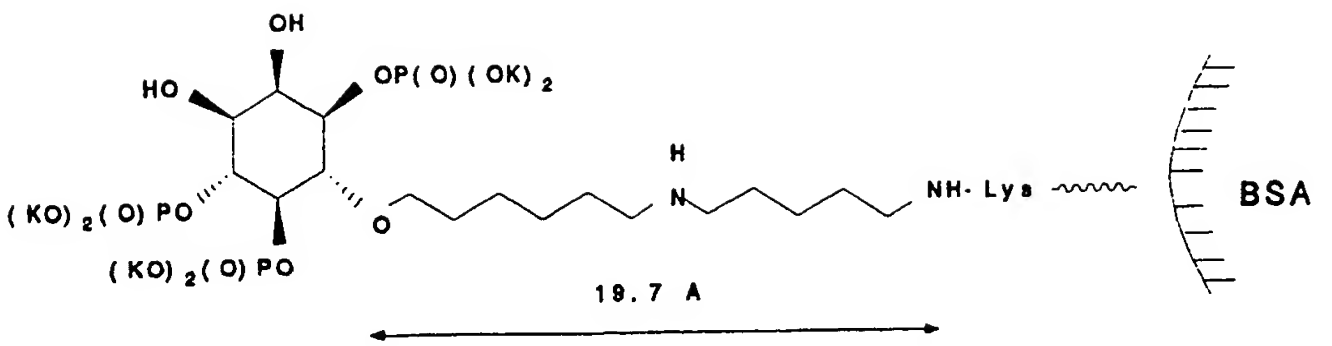
The intricate role of Ins(1,4,5)P_3 in modulating intracellular Ca^{2+} homeostasis has been the focus of many recent investigations, Berridge, M.J. *Nature*, 1993, **361**, 315, and references cited therein. It is well documented that the initial agonist-induced Ca^{2+} release is derived from the Ca^{2+}-mobilizing action of Ins(1,4,5)P_3 on intracellular organelles whose exact nature is still in dispute, Meldolesi, J.; Villa, A.; Volpe, P.; Pozzan, T. *Advances in Second Messenger and Phosphoprotein Research*; Putney, J.W., Jr., Ed.; Raven Press: New York, 1992; Vol. 26, pp 187-208. While many researchers assert that IP_3-specific receptors distribute mainly on the membrane of the endoplasmic reticulum or nucleus, others suggest the locality on a more specialized endomembrane fraction, i.e., calciosomes, Volpe, P.; Kraus, K.H.; Sadamitsu, H.; Zorzato, F.; Pozzan, T.; Meldolesi, J.; Low, P.B. *Proc. Natl. Acad. Sci. USA*, 1988, **85**, 1091-1095. These Ins(1,4,5)P_3-specific receptors are thought to function as a ligand-gated Ca^{2+} channel, Gill, D.L.; Ghosh, T.K.; Bian, J.; Short, A.D.; Waldron, R.T.; and Rybank. S.L., *Advances in Second Messenger and Phosphoprotein Research*, 1992, Vol. 26, (Putney, J.W. ed), Raven Press, New York; pp. 265-308. In the second phase of the signaling process, i.e., Ca^{2+} entry from the extracellular medium, Ins(1,4,5)P_3 has also been implicated. The capacitative entry theory suggests that depletion of the intracellular Ca^{2+} store by Ins(1,4,5)P_3 generates a secondary signal of unknown nature that activates Ca^{2+} entry, Putney, J.W., Jr. *Cell Calcium*, 1986, **7**, 1-12; Takemura, H., and Putney, J.W., Jr. *Biochem. J.*, 1989, ***258***, 409-421; and Putney, J.W., Jr.; Takemura, H.; Hughes, A.R.; Horstman, D.A.; and Thastrup, O. (1989) *FASEB J.* **3**, 1899-1905. Thus, an important issue to be addressed is the mechanism of interaction between the Ins(1,4,5)P_3-sensitive Ca^{2+} pool and the plasma membrane.

The present invention in one aspect embodies novel Ins(1,4,5)P_3 analogues and the syntheses for such analogues. Although a number of Ins(1,4,5)P_3 analogues have been

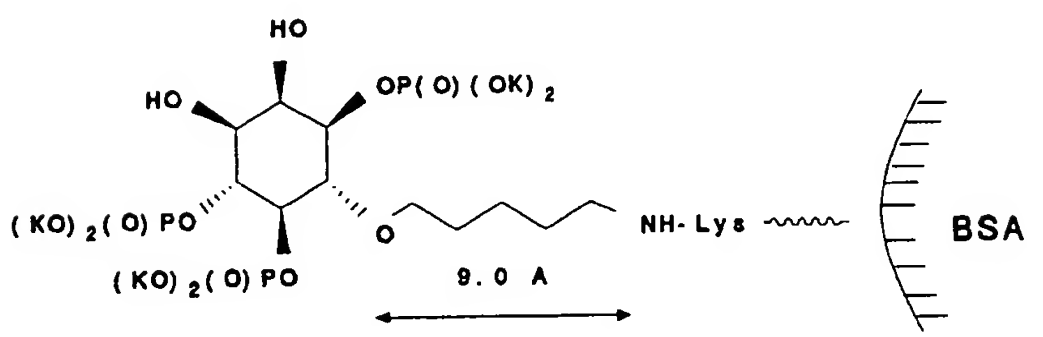
reported, the reactive appendages of these derivatives were attached to the parent molecule through a C-1 phosphodiester linkage, Prestwich, G. D.; Marecek, J. F.; Mourey, R. J.; Theibert, A. B.; Ferris, C. D.; Danoff, S. K.; Snyder, S. H., *J. Am. Chem. Soc.*. 1991, 113, 1822; Tegge, W.; Ballou, C. E., *Carbohydr. Res.*, 1992, 230, 63. The present invention, in a preferred embodiment, is directed to the synthesis of two novel Ins(1,4,5)P_3 analogues, **1** and **2**, with modification at the C-6 position.

The derivatized Ins(1,4,5)P_3 contains an amine or aldehyde function for further elaborations which allows the preparation of Ins(1,4,5)P_3-based analogues for antibody induction, affinity purification, and histochemical probing.

In researching inositol phosphate-mediated Ca^{2+} homeostasis, our effort has focused on the synthesis of endogenous inositol polyphosphates and the generation of anti-Ins(1,4,5)P_3 antibodies in view of their potential use as biological probes. Although antibodies to PIP and PIP_2 have been prepared through immunizing animals with inositol-phospholipids, Matuoka, K.; Fukami, K.; Nakanishi, O.; Kawai, S.; and Takenawa, T. (1988) *Science* **239**, 640-643, or with those liposomes containing these compounds, Roerdink, F.; Berson, B.J.; Richard, R.L.; Swartz, G.M., Jr.; Alving, C.R. (1980) *Fed Proc.* **40**, 996; Freidman, R.L.; Iglewski, B.H.; Roerdink, F.; and Alving, C.R. (1982) *Biophys. J.* **37**, 23-24; Fukami, K.; Matsuoka, K.; Nakanishi, O.; Yamakawa, A.; Kawai, S.; and Takenawa, T. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 9057-9061; and Bate, C.A.W.; Taverne, J. Bootsma, H.J.; Mason, R.C. St. H.; Skalko, N.; Gregoriadis, G.; and Playfair, H.L. (1992) *Immunology* **76**, 35-41, a similar strategy could not be employed for inducing anti-Ins(1,4,5)P_3 antibodies because of the vast difference in their water solubilities. Hence, the invention in another aspect broadly embodies the preparation of highly specific anti-Ins(1,4,5)P_3 antibodies using two types of Ins(1,4,5)P_3-BSA conjugates shown below as antigens. The analogues shown in the conjugates below are the Ins(1,4,5)P_3 analogues 1 and 2 mentioned above.



I P_3- C_6NC_6- BSA



I P_3- C_6- BSA

The invention also embodies an affinity matrix for purifying Ins(1,4,5)P_3-specific antibodies and a unique ELISA system.

Brief Description of the Drawings

Fig. 1 is a graph of Ca^{2+} releasing activity of Ins(1,4,5)P_3 versus analogue 1;

Fig. 2 is a graph of the affinity purification of Ins (1,4,5, P_3-specific antibodies);

Fig. 3 is a graph of Inositol phosphate specificity of affinity-purified antibodies; and

Fig. 4 is a graph of the inhibition of antibody binding

to immobilized Ins(1,4,5) P_3 by multivalent anions.

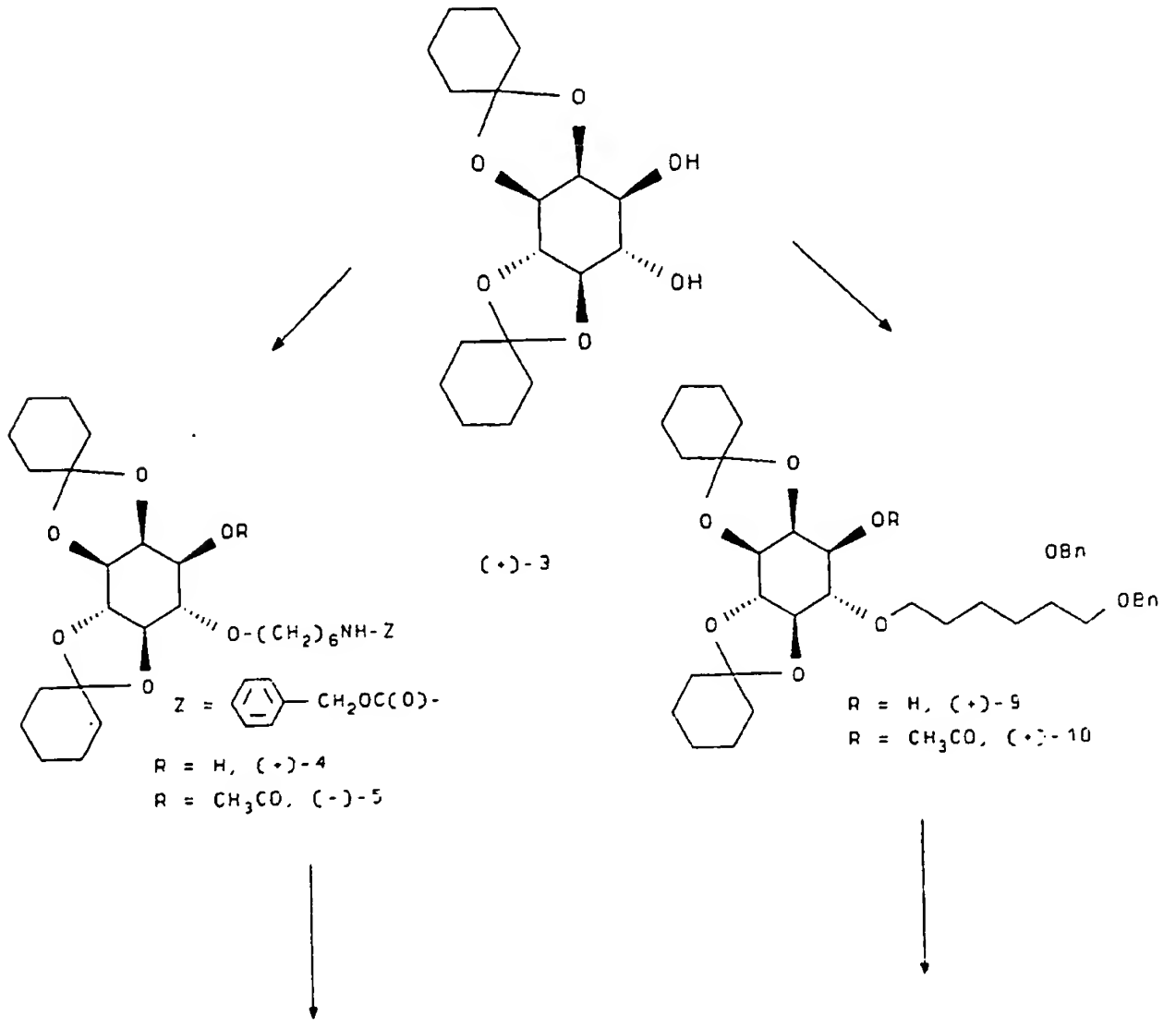
Description of the Preferred Embodiment(s)

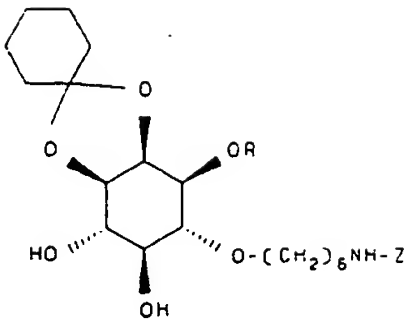
The references cited in the following sections are hereby incorporated by reference in their entireties into this disclosure.

Analogues

In light of the delicate structure of Ins(1,4,5)P_3, the latitude in the choice of a site(s) for modification is limited. The design of these two optically active Ins(1,4,5)P_3 analogues, **1** and **2**, was based on (a) the strategic importance of the C-2,3 cis-dihydroxy moiety, especially the axial 2-OH, in recognizing Ins(1,4,5)P_3 versus other inositol phosphates, and (b) the intactness of the three phosphate functions in derivatized Ins(1,4,5)P_3 to achieve optimal ionic interactions with the binding proteins.

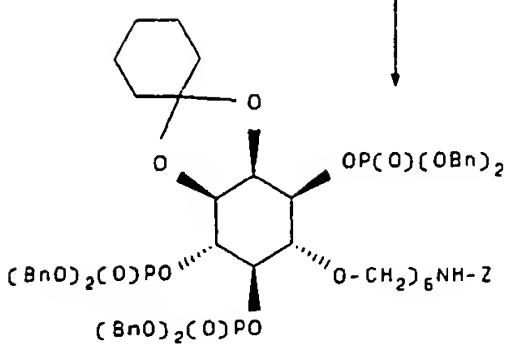
Both **1** and **2** were synthesized from (+)-2,3:4,5-di-O-cyclohexylidene-D-myoinositol, (+)-**3**, through similar routes.



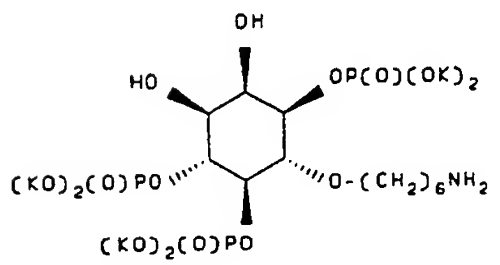


R = CH_3CO, (-)-6

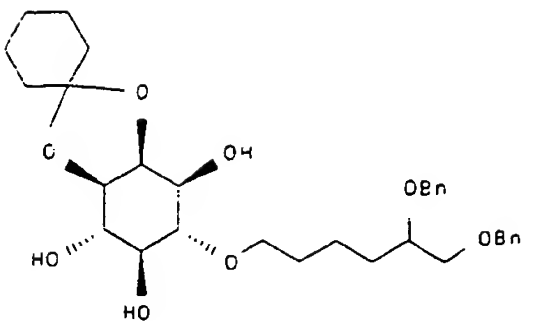
R = H, (+)-7



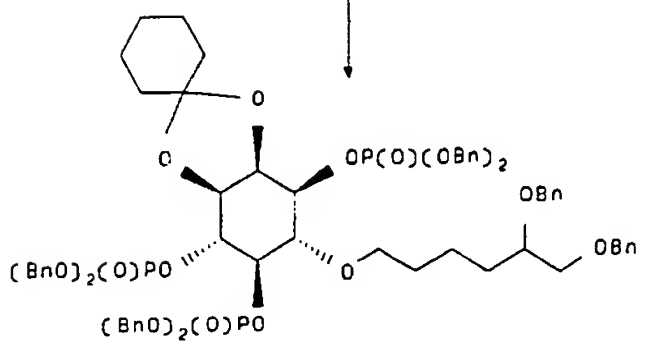
(-)-8



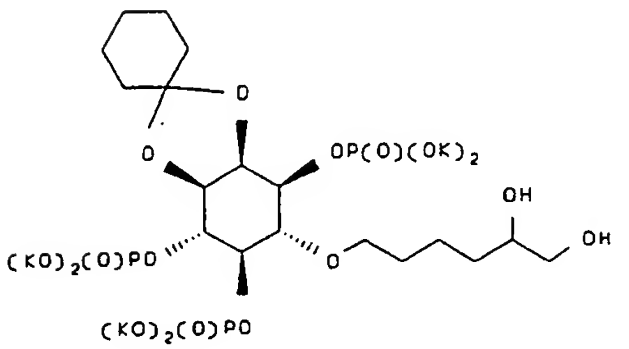
(-)-1



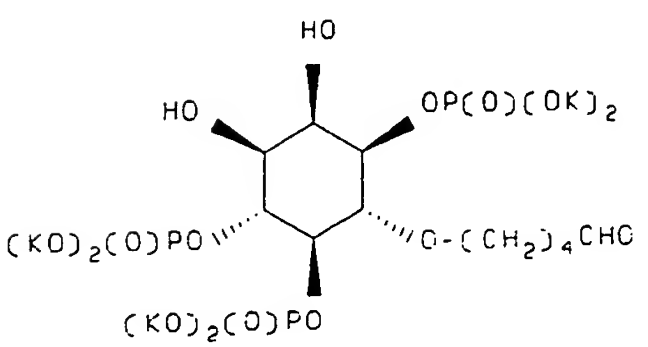
(+)-11



(-)-12



(-)-13



(-)-2

The chiral precursor was prepared by enantioselective hydrolysis of its racemic 6-O-butyryl ester by porcine pancreatic lipase, Gou, D.-M; Liu, Y.-C.; Chen, C.-S., *Carbohydr. Res.*, 1992, 234, 51. The stannylidene-activated alkylation allowed the regioselective introduction of the substituents at the 6-O position to afford (+)-**4** and (+)-**9** in good yields. To prevent acid-catalyzed migration of the cis-cyclohexylidene group under acidic conditions, **4** and **9** were acetylated to give (-)-**5** and (+)-**10**, respectively, methanolysis of which removed the trans-cyclohexylidene ring and saponification of the products furnished (+)-**7** and (+)-**11**, respectively. Phosphorylation of the triol **7** by the phosphoramidite method, Yu, K.-L.; Fraser-Reid, B., *Tetrahedron Lett.*, 1988, 29, 979, followed by debenzylation and acid hydrolysis of the cisketal, gave the trisphosphate (-)-**1**. Similar treatments of **11** but without the acid hydrolysis led to (-)-**13**. The overall yields for the synthesis of **1** and **13** from (+)-**3** were 37% and 45%, respectively. These two compounds were fully characterized by ^{1}H and ^{31}P NMR and mass spectrometry. The aldehyde-bearing derivative (-)-**2** was readily afforded by subjecting the vicinal diol **13** to periodate oxidation and a subsequent acid treatment.

With the functionalities of amine and aldehyde in the side arms, analogues **1** and **2** allows the coupling of Ins(1,4,5)P_3 to virtually any type of molecule. Moreover, the three phosphate functions of the resulting conjugates are fully exposed, resembling the charge state of the parent compound.

The derivatized Ins(1,4,5)P_3 retained the biological activity of Ins(1,4,5)P_3. For instance, **1** was able to mobilize 80% of Ins(1,4,5)P_3-sensitive Ca^{2+} store when porcine brain microsomes were saturated. Referring to Fig. 1, Ca^{2+}-loaded porcine brain microsomes were treated with Ca^{2+}-mobilizing agents, and released Ca^{2+} was monitored by a Ca^{2+}-sensitive fluorescent dye, Fura-2, according to the method described in the experimental section (100% = Ca^{2+} release at saturated concentrations of Ins(1,4,5)P_3). Each data point represents

the means of three determinations.

The EC_{50} values were 79 nM and 18 μM for Ins(1,4,5)P_3 and **1**, respectively. Although the Ca^{2+}-mobilizing activity of the analogue was about 200-fold less potent than that of the parent compound, it could interact functionally with the Ins(1,4,5)P_3-specific receptor(s) in the microsomes. The decrease in affinity was presumably due to the hydrophobic side arm which imposed steric and/or stereoelectronic effect(s) on binding. It has recently been reported that the 6-OH may have an important role in the interactions with receptors and metabolis enzymes, Hirata, M.; Watanabe, Y.; Yoshida, M.; Koga, T.; Ozaki, S., *J. Biol. Chem.*, 1993, 268, 19260; Kozikowski, A. P.; Ognyanov, V. I.; Fauq, A. H.; Nahorski, S. R.; Wilcox, R. A., *J. Am. Chem. Soc.*, 1993, 115, 4429. The finding that these 6-O substituted analogues were active in Ca^{2+} mobilization broadened the utility of these molecules, which is illustrated in the following two examples.

Materials. (1,4,5)IP_3, (1,3,4)IP_4, and (1,3,4,5)IP_4 were synthesized from optically active 1,2:5,6-di-O-cyclohexylidene- inositol **1** (optical purity > 98% enantiomeric excess) according to previously described procedures, Gou, D.-M; Liu, Y.-C.; Chen, C.-S., *Carbohydr. Res.*, 1992, 234, 51; Liu, C.-Y.; Chen, C.-S., *Tetrahedron Lett.*, 1989, 30, 1617; Gou, D.-M.; Chen, C.-S.;, *Tetrahedron Lett.*, 1992, 33, 721. (4,5)IP_2, (1,5,6)IP_3 were prepared by following a similar approach with pertinent modifications. The chemical purity of these chiral inositol phosphates was greater than 95% according to ^1H and ^{31}P NMR spectroscopy. The amount of isomeric impurities was negligible as indicated by these NMR spectra. Phytic acid was purchased from Sigma.

(+)-6-O-(ω-Benzyloxycarbonylaminohexyl)-2.3:4,5-di-O-cyclohexylidene-myo-inositol **4** - A mixture of (+)-2,3:4,5-di-O- cyclohexylidene-D-myo-inositol **3** (1 g, 2.9 mmol), Bu_2SnO (840 mg, 3 mmol), and toluene (25 ml) was boiled under reflux with azeotrophic removal of water for 1 h, then concentrated to dryness under reduced pressure. To the residue were added N,N-dimethylformamide (10 ml) and -

benzyloxycarbonylaminohexyl bromide (2 g, 8.5 mmol). The mixture was stirred at 23 °C overnight, then diluted with CH_2Cl_2(50 ml), washed with saturated aq. NaCl, dried with Na_2SO_4, and concentrated. Column chromatography of the residue on a silica gel column (hexane-ether, 15: 1) gave **4** (1.1 g, 65%). [α]D +4-9° (c 1.7, CHC13). ^{1}H NMR ($CDCl_3$) δ 1.25 - 1.71 (m, 28 H), 2.66 (d, 1 H, J= 1 Hz), 3.14 - 3.21 (m, 2 H), 3.40 - 3.48 (m, 1 H), 3.50-3.59 (m, 1 H), 3.63 -3.73 (m,2H), 3.93-3.94 (m, 1 H), 4.12-4.18 (m, 1 H), 4.32 (t, 1 H, J = 6 Hz), 4.37 - 4.41 (m, 1 H), 4.86 (m, 1 H), 5.08 (s, 2 H), 7.28 - 7.35 (m, 5 H); 13C NMR ($CDCl_3$) δ 15.16, 23.37, 23.65, 23.73, 25.03, 25.09, 25.69, 26.42, 29.59, 29.87, 33.52, 36.35, 36.57, 36.67, 41.07, 53.28, 65.69, 66.53, 69.78, 72.35, 75.34, 76.74,77.19,78.19,78.84,80.40, 111.17, 112.71, 127.96, 128.42, 136.79, 156.39.

(-)-1-0-Acetyl6-0-(ω-benzyloxycarbonylaminohlexyl)-2,3-O-cyclohexylidene-myoinositol **6** - A solution of **4** (1 g, 1.7 mmol) in CH_2Cl_2 (10 ml) was treated with acetic anhydride (0.4 ml, 3.5 mmol), and 4-dimethylaminopyridine (208 mg, 1.7 mmol) at 23 °C for 30 min. The mixture was washed with aq. $NaHCO_3$ and water, dried, and concentrated to afford (-)-1-O-acetyl-6-O-(benzyloxycarbonylaminohexyl)-2,3:4,5-di-O-cyclohexylidene-myo-inositol **5** (syrup) in quantitative yield. 13C NMR ($CDCl_3$) δ 13.97, 15.18, 21.10, 22.57, 23.49, 23.70, 23.87, 25.03, 25.09, 25.65, 26.42, 29.54, 29.89, 3 1.52, 34.27, 36.47, 36.63, 41. 12, 64. 10, 65.72, 66.54, 70.01, 72.81, 73.41, 77. 19, 78.38, 79.75, 111.62, 113.02, 127.99, 128.45, 136.86, 156.41, 169.43. A solution of the oily compound in CH_2Cl_2-CH_3OH (1:1, 10 ml) was stirred with acetyl chloride (0.1 ml, 1.2 mmol) at 23 °C for 10 min. Triethylamine (0.3 ml) was added, and the solution was concentrated. Column chromatography (hexane-ether, 20: 1) of the residue yielded (-)-**6** (syrup, 0.67 g,75%). [α]D -14.8° (c 1.1, $CHCl_3$). ^{1}H NMR ($CDCl_3$) δ 1.30 - 1.72 (m, 18 H), 2.14 (s,3H), 3.15-3.21 (m,2H), 3.33 -3.39 (m, 1 H),3.44-3.51 (m,2H), 3.56 (t, 1 H,J= 9 Hz), 3.64 - 3.78 (m, 3 H), 4.01 - 4.05 (m, 1 H), 4.39 - 4.42 (m, 1 H), 4.85 (m, 1 H), 5.03 - 5.09 (m,3 H), 7.30 - 7.36 (m, S H); ^{13}C NMR ($CDCl_3$) δ 13.97, 15.16, 21.02, 23.56, 23.97,

25.03, 25.65, 26.35, 29.85, 29.97, 34.99, 37.58, 66.67, 71.64, 72.48, 73.17, 73.70, 74.92, 77.19, 78.30, 79.49, 110.97, 113.02, 127.99, 128.04, 128.50, 156.52, 170.02.

(+)-6-O-(ω-benzylocycarbonylaminohexyl)-2,3-O-cyclohexlidene-myo-inositol **7** - The acetate **6** (0.9 g, 1.7 mmol) was treated with methanolic 1 M NaOH for 1 h at 23 °C, and the solvent was evaporated under reduced pressure. Column chromatography (hexane-ether, S: 1) of the residue afforded (+)-**7** (0.77 g, 93%). $[\alpha]D$ +11.6° (c 2.4, $CHCl_3$). ^1H NMR ($CDCl_3$) δ 1.34 - 1.72 (m, 18 H), 2.84 (br. s, 1 H), 3.13 - 3.20 (m, 2 H), 3.30-3.35 (m, 1 H), 3.44-3.51 (m, 2 H), 3.69-3.85 (m, 5 H), 3.99-4.03 (m, 1 H), 4.34 - 4.37 (m, 1 H), 5.01 (br. s, 1 H), 5.08 (br. s, 2 H), 7.27 - 7.35 (m, 5 H); ^{13}C NMR ($CDCl_3$) δ 23.61, 24.03, 25.08, 25.66, 26.38, 29.88, 30.00, 34.75, 37.52, 41.07, 66.71, 70.47, 72.16, 74.04, 75.08, 75.26, 77.10, 78.29, 81.90, 110.73, 128.09 (2C), 128.54 (2C), 136.83, 156.64.

6-O-(ω-benzyloxycarbonylaminohexyl)-2,3-O-cyclohexylidene-myo-inositol 1,4,5-tris-(dibenzyl phosphate) **8** - A mixture of 1H-tetrazole (2.5 g, 35 mmol), dibenzyl N,N-di-isopropyl-phosphoramidite (6 g, 7 mmol), and CH_2Cl_2 (40 ml) was stirred under Ar at 23 °C for 1 h, and (+)-**7** (0.75 g, 1.5 mmol) was added in one portion. The solution was kept under the same conditions for another 12 h, cooled to - 15 °C, and then treated with m-chloroperoxybenzoic acid (50% purity, 7 g, 17 mmol). The mixture was stirred at -15 °C for 30 min, then allowed to attain room temperature, diluted with CH_2l_2 (80 ml), washed with aq. Na_2SO_3, aq. $NaHCO_3$, and water, dried, and concentrated. Column chromatography (hexane-ether, 15:1) of the residue furnished (-)-**8** (syrup, 1.6 g, 83%). $[\alpha]_D$ -3.4° (c 3, $CHCl_3$). ^1H NMR ($CDCl_3$) δ 1.11 - 1.74 (m, 18 H), 3.08 (q, 2 H, J=6.5 and 12.9 Hz), 3.43 -3.48 (m, 1 H), 3.54-3.60 (m, 1 H), 3.97 (q, 1 H, J = 5.4 and 7.8 Hz), 4.24 (t, 1 H, J = 6.6 Hz), 4.48 - 4.58 (m, 2 H), 4.67 - 4.74 (m, 1 H), 4.87-5.10 (m, 16H), 7.20-7.32 (m,35H);

(-)6-O-(ω-aminohexyl)-D-myo-inositol 1,4,5-triphosphate **1** - A solution of (-)-**8** (1.5 g, 1.2 mmol) in aq. 80% ethanol

was shaken under H_2 (50 psi) in the presence of 10% Pd/C (0.5 g) for 15 h, then filtered, and concentrated to dryness. To the residue was added 2 ml of glacial acetic acid, and the resulting solution was stirred at 23 °C for 2 h, and concentrated. The residue was triturated with CH_2Cl_2-ether (1:1), the resulting white precipitate was dissolved in the minimum amount of water, and 1 M KOH (6 equiv.) was added. The solution was lyophilized to afford **1** as the hexapotassium, acetate salt (0.92 g, 99%). $[\alpha]_D$ -14° (c 1.1, H_2O). ^1H NMR (D_2O) δ 1.10 - 1.23 (m, 4 H), 1.23 - 1.38 (m, 2 H), 1.40 - 1.53 (m, 2 H), 2.48 (br. s, 2 H), 3.43 - 3.72 (m, 4 H), 3.91 - 4.14 (m, 4 H); ^{31}P NMR (D_2O, external H_3PO_4, broadband decoupled): 2.28, 2.64, and 3.18. FAB-MS m/q 790 [$C_{12}H_{22}NO_{15}P_3K_6$ (M) - H2O], 752 M - K + 1H], 714 [M - 2K + 2H], 672 [M - 2K - HOAc + 2H], 591 [M- P(O)$(OK)_2$].

(+)-6-0-(5',6'-dibenzyloxyhexyl)-2:3,4:5-di-0-cyclohexylidene-myo-inositol **9** - A mixture of **3** (1 g, 2.9 mmol), Bu_2SnO (840 mg, 3 mmol), and toluene (30 ml) was boiled under reflux for 1 h, then concentrated to dryness under reduced pressure. To the residue were added N,N-dimethylformamide (10 ml), CsF (1.3 g, 8.6 mmol), and 5,6 dibenzyloxyhexyl bromide (2.5 g, 6.7 mmol). The mixture was stirred at 23 °C overnight, then diluted with ethyl acetate (50 ml), washed with saturated aq. NaCl, dried (Na_2SO_4), and concentrated. Column chromatography (hexane-ether, 15:1 ---> 5:1) of the residue gave **9** (syrup, 1.5 g, 80%). $[\omega]_D$ +3.8° (c 3, CHC_3). ^1H NMR ($CDCl_3$) 1.38 - 1.72 (m, 26 H), 2.59 - 2.63 (m, 1 H), 3.40 - 3.73 (m, 7 H), 3.90 - 3.93 (m, 1 H), 4.12 - 4.19 (m, 1 H), 4.29 - 4.38 (m, 2 H), 4.53 - 4.71 (m, 4 H), 7.26 - 7.35 (m, 10 H); ^{13}C NMR ($CDCl_3$) δ 22.08, 23.49, 23.77, 23.85, 23.94, 25.15, 25.21, 29.90, 31.88, 33.63, 36.47, 36.70, 36.79, 69.89, 72.05, 72.06, 72.46, 72.47, 73.09, 73. 1 1, 73.48, 75.45, 76.87, 77.26, 78.27, 78.98, 79.00, 80.47, 111.28, 112.82, 127.46, 127.59, 127.64, 127.79, 128.31, 128.40. 138.61. 139.17.

(+)-6-0-(5',6'-dibenzyloxyhexyl)-2:3-0-cyclohexylidene-myo-inositol **1 1** - A solution of **9** (1.5 g, 2.3 mmol) in CH_2Cl_2 (15 ml) was treated with acetic anhydride (1.2 ml, 12.7 mmol)

and 4-dimethylaminopyridine (1.6 g, 13 mmol) at 23 °C for 1 h. The mixture was washed with aq. $NaHCO_3$ and water, dried, and concentrated to afford 1-O-acetyl-6-O-(5',6'-dibenzoxyhexyl)-2:3-cyclohexylidene-myo-inositol (**10**) in quantitative yield. Without purification, the oily compound was dissolved in a solution of $CH_2Cl_2CH_3OH$ (1:1, 10 ml), and treated with acetyl chloride (0.1 ml, 1.4 mmol) for 10 min. Triethylamine (0.5 ml) was added, and the solution was concentrated. To the crude residue was added 1.2 equiv. of methanolic 1 M NaOH. The solution was stirred at 23 °C for 1 h, and solvent was removed under reduced pressure. Column chromatography (hexane-ether, 10: 1) of the residue afforded **11** (syrup, 0.91 g, 70%). $[\alpha]_D$ +9.9° (c 1.1, $CHCl_3$). ^1H NMR ($CDCl_3$) δ 1.30 - 1.74 (m, 16 H), 2.80 (dd, 1 H, J = 4.8 and 60 Hz), 3.12 - 3.32 (m, 3 H), 3.38 - 3.44 (m, 1 H), 3.49 - 3.59 (m, 4 H), 3.68 - 3.74 (m, 4 H), 3.90 -3.98 (m, 1 H), 4.19-4.34 (m, 1 H), 4.50-4.55 (m, 2 H), 4.70 (dd, 1 H, J=4.8 and 10 Hz), 7.26 - 7.34 (m, 10 H); ^{13}C NMR ($CDCl_3$) δ 21.66, 21.71, 23.58, 23.99, 25.05, 29.92, 31.49, 34.81, 34.84, 37.56, 70.49, 71.94, 71.95, 72.17, 72.24, 72.91, 72.96, 73.48, 73.79, 75.19, 75.29, 78.20, 78.29, 81.49, 81.67, 127.60, 127.64, 127.69, 127.97, 128.04, 128.36, 128.41.

(-)-6-O-(5',6'-dibenzyloxyhexyl)-2:3-O-cyclohexylidene-myo-inositol 1,4,5-tris (dibenzyl phosphate) **12** - A mixture of 1H-tetrazole (2.75 g, 39 mmol), dibenzyl N, N-di isopropylphosphoramidite (7.49 g, 8.7 mmol), and CH_2Cl_2 (30 ml) was stirred under Ar at 23 °C for 1 h, and (+)-**11** (0.89 g, 1.6 mmol) was added in one portion. The solution was kept under the same condition for another 12 h, cooled to 40 °C, and then treated with m-chloroperoxybenzoic acid (50% purity, 9 g, 25 mmol). The mixture was stirred at -40 °C for 10 min, then allowed to attain room temperature, diluted with CH_2Cl (80 ml), washed with aq. Na_2SO_3, aq. $NaHCO_3$, and water, dried, and concentrated. Column chromatography (hexane-ether, 15: 1) of the residue furnished (-)-**12** (syrup, 2 g, 93%). $[\alpha]_D$ -3° (c 1.1, $CHCl_3$). ^1H NMR data ($CDCl_3$): δ 1.25 - 1.91 (m, 16H), 3.38-3.60 (m, 5 H), 3.98 (dd, 1 H, J=4.8 and 6.0Hz), 4.25 (t,

1 H, J = 6.6 Hz), 4.47-4.70 (m, 6 H), 4.92-5.10 (m, 14 H), 7.21-7.34 (m, 40 H).

6-0-(5',6'-dihyroxyhexyl)-2:3-O-cyclohexylidene-myo-inositol 1,4,5-triphosphate **13** - A solution of **12** (0.2 g, 0.15 mmol), and 1 M KOH (0.9 ml, 0.9 mmol) in aq. 80% ethanol was shaken under H_2 (50 psi) in the presence of 10% Pd/C (1 g) for 24 h, then filtered, and concentrated. The residue was dissolved in water (10 ml), and lyophilized to afford **13** as the hexapotassium salt (0.13 g, 99%). ^1H NMR data (D_2O): δ 1.15 - 1.76 (m, 16 H), 3.25 - 3.38 (m, 1 H), 3.40 - 3.51 (m, 1 H), 3.52 - 3.80 (m, 3 H), 4.06 - 4.18 (m, 1 H), 4.23 - 4.35 (m, 2 H), 4.42 - 4.55 (m, 2 H), 4.65 - 4.80 (m, 1 H). ^{31}P (D_2O, external H_3PO_4, broadband decoupled): 3.44, 4.11, and 4.75. FAB-MS m/q 845 [$C_{18}H_{29}O_{17}P_3K_6$ (M)], 807 [M - K + 1H], 769 [M - 2K + 2H].

Materials

6-O-(ω-Aminohexyl)-D-*myo*-inositol 1,4,5-triphosphate **1** and 6-O-5',6'-dihydroxyhexyl)-2:3-O-cyclohexylidene-D-*myo*-inositol 1,4,5-triphosphate **2** were used for the preparation of IP_3-C_6NC_5-BSA and IP_3-C_5-BSA respectively. Both compounds were extensively characterized by ^1H and ^{31}P NMR and FAB mass spectrometry. Their synthesis is described hereinafter.

Ins(1,4,5)P_3, Ins(1,3,4)P_3, and Ins(1,3,4,5)P_4 were synthesized from optically active 1,2:5,6-di-O-cyclohexylidene-inositol (optical purity > 98% *e.e.*) as described above and according to previously described procedures, Liu, Y.-C.; and Chen, C.-S. (1989) *Tetrahedron Lett.* **30**, 1617-1620; Gou, D.-M.; and Chen, C.-S. (1992) *Tetrahedron Lett.* **33**, 721-724; and Gou, D.-M; Liu, Y.-C; and Chen, C.-S. (1992) *Carbohydr. Res.* **234**, 51-64. Ins(4,5)P_2, Ins(1,5,6)P_3, Ins(1,2,5,6)P_4 were synthesized by following the same procedures with the following modifications. The chemical purity of these chiral inositol phosphates was greater than 98% according to ^1H and ^{31}P NMR spectroscopy. The amount of

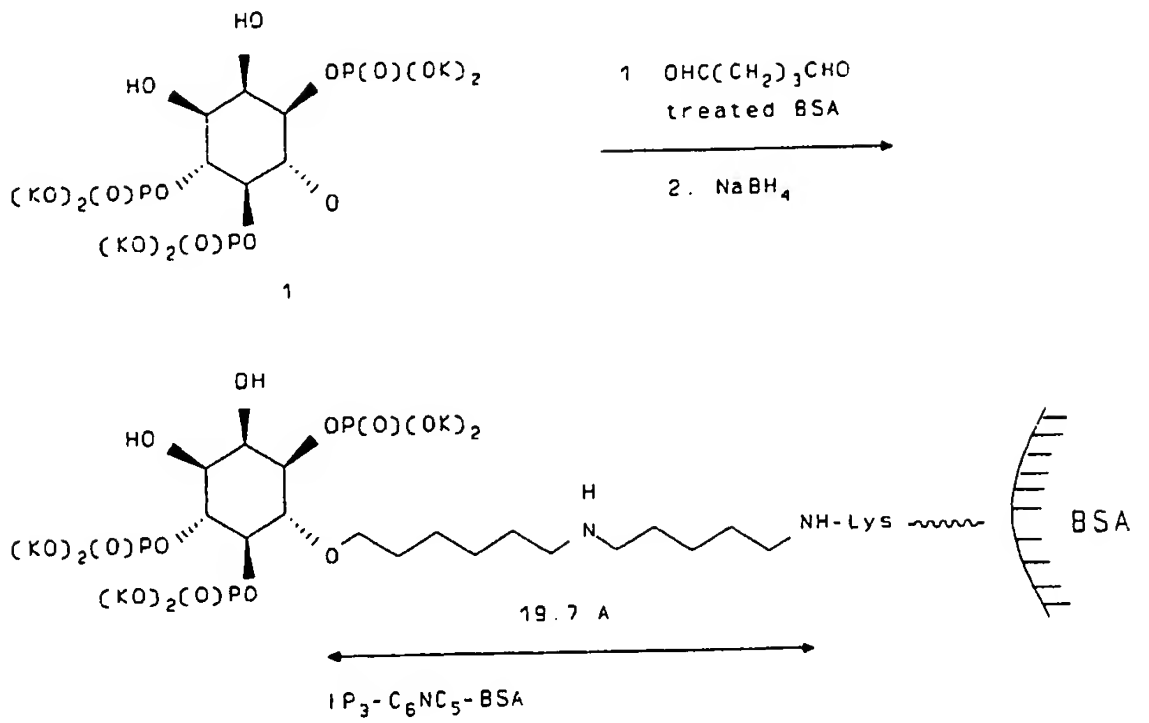
isomeric impurities was not detectable as indicated by these NMR spectra. Phytic acid, PIP_2, and $Ins(1)P_1$ were purchased from Sigma. Other chemicals and biochemicals were supplied from Sigma or Aldrich unless otherwise mentioned.

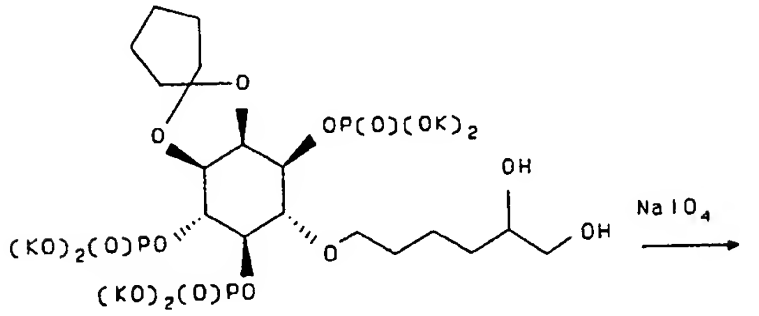
General Methods ^1H, ^{13}C, and ^{31}P NMR spectra were recorded with a Bruker AM300 spectrometer. Optical rotations were determined at 23 °C with a Rudolph Autopol III polarimeter. Fast atom bombardment mass spectra (FAB-MS) were obtained from the Chemical Instrument Center, Yale University. Elemental analysis was performed by MH-W laboratories (Phoenix, AZ). Fluorescence spectrophotometric assay of Ca^{2+} release was carried out with a Hitachi F-2000 spectrometer.

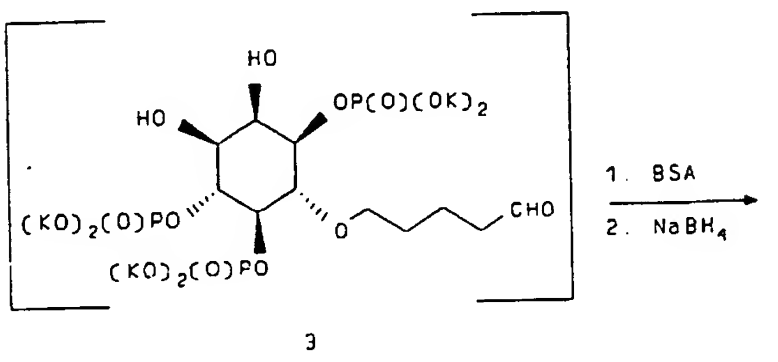
Preparation of $Ins(1,4,5)P_3$-agarose

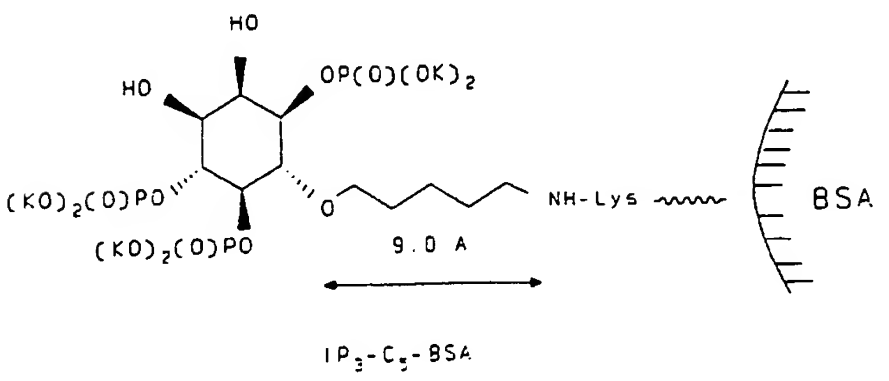
$Ins(1,4,5)P_3$-agarose was prepared by reacting **1** with 1,1'-carbonyldiimidazole-activated 6% crosslinked beaded agarose (Reacti-Gel®; Pierce) according to the standard protocol recommended by Pierce Chemical Co. Instruction 20259 (1989) Reacti-Gel® (6X). In brief, activated Reacti-Gel® (6 ml) was thoroughly washed with distilled water, and was added to 10 mM borate buffer, pH 9.5, (15 ml) containing the IP_3 ligand (28 mg). The suspension was incubated at room temperature with gentle shaking for 25 hours. The reaction was terminated by adding 10 ml of 1 M Tris/HCl, pH 8.0, to the reaction mixture. The gel was recovered by filtration, thoroughly washed, and stored in 10 mM Tris/HCl, pH 7.5, containing 0.1% NaN_3 at 4°C.

Preparation of IP_3-BSA conjugates and immunization









IP_3-C_6NC_5-BSA. 10% Glutaraldehyde (4 ml) was added dropwise to a solution of BSA (20 mg) in 10 mM $NaHCO_3/Na_2CO_3$ buffer, pH 9.5. The mixture was stirred at 5°C for 40 minutes, and dialyzed against the same buffer overnight with at least three changes. **1** (20 mg) was then added, and the mixture was incubated at 15°C. After 3 hours, $NaBH_4$ (100 mg) was added, and the incubation continued at 15°C for additional 3 hours. The solution was dialyzed against 10 mM $NaHCO_3/CO_2$ buffer, pH 7.0, overnight, and lyophilized to afford IP_3-C_6NC_5-BSA.

IP_3-C_5-BSA. $NaIO_4$ (12 mg, 0.06 mmol), dissolved in water (1 ml), was added in several portions to a solution of **2** (50 mg, 0.06 mmol) in distilled water (1 ml), pH 7.5, at 0°C. The mixture was stirred at 20°C for 2.5 hours, and 1 N HCl (100 µl) was added. After stirring for an additional 2 hours, the solution was extracted with ethyl ether to remove cyclohexanone, adjusted to pH 8 with $NaHCO_3$, and lyophilized to afford 6-O-(4'-formylbutyl)-*D*-myo-inositol 1,4,5-triphosphate **3**. The aldehyde intermediate, without purification, was incubated with BSA (20 mg) in 4 ml of 10 mM $NaHCO_3/Na_2CO_3$ buffer, pH 9.5, at 5°C for 2 hours. $NaBH_4$ (20 mg) was then added, and the incubation continued at room temperature for an additional 2 hours. The reaction mixture was dialyzed against 10 mM $NaHCO_3/CO_2$ buffer, pH 7.4, overnight, and lyophilized to give IP_3-C_5-BSA.

Immunization

New Zealand White female rabbits were immunized with 1 mg of the IP_3-BSA conjugate in 0.5 ml of saline emulsified with an equal volume of Freund's complete adjuvant by five to six subcutaneous injections at the back and both flanks. Booster injections were given with the same amounts of the IP_3-BSA prepared with incomplete Freund's adjuvant at monthly intervals. After the second boost, the animals were bled from the ear vein 2 weeks after each booster injection. The antisera thus prepared were stored at -20°C until used.

ELISA

The presence of anti-Ins(1,4,5)P_3 antibodies in rabbit serum was detected by an ELISA. Ins(1,4,5)P_3 (hapten) was covalently attached to microtiter plates through a C-6 linker by adding 6-O-(ω-Aminohexyl)-*D*-myo-inositol 1,4,5-triphosphate (**1**) (1.25 μg in 0.1 ml of phosphate buffered saline (PBS) per well) to maleic anhydride-activated polystyrene plates (Pierce). This anhydride-activated plate allows direct coupling of amine-bearing molecules to the well surface, (Pierce Chemical Co. Instruction 15110X (1992) Reacti-Bind™ Maleic Anhydride Activated Polystyrene Plates), and obviates the need for a carrier protein to immobilize haptens. The plate was incubated at room temperature overnight, and each well was blocked by adding 0.12 ml of SuperBlock blocking solution in PBS (Pierce). After 10 minutes at room temperature, the plate was washed twice with 0.05% Tween-20 in 10 mM Tris/HCl containing 0.85% NaCl, pH 7.2 (TBS), followed by TBS buffer twice. Rabbit antiserum (antibodies) diluted in TBS buffer containing 0.1% gelatin was added (95 μl per well). The plate was incubated at 37°C for 2 hours, and washed as stated above. Specific antibody binding was assessed by adding goat anti-rabbit IgG-HRP conjugate (1/200 dilution in TBS containing 0.1% gelatin; 90 μl per well), and the plate was incubated for another 2 hours at 37°C. After the plate was washed again, the peroxidase substrate solution (90 μl per well) containing 2 mM 2,2'-azinobis(3-ethyl-benzthiazoline-6-sulfonic acid), 2.5 mM hydrogen peroxide, and 50 mM citrate buffer, with a final pH of 5.0, was added. The reaction was incubated at room temperature for 15-20 minutes, and terminated by adding 20 μl of 5% SDS to each well. The absorbance at 415 nm was measured by a microtiter plate reader. Rabbit serum taken before immunization was used as a control in all the assays, from which the absorbance values obtained served as a blank for the correction of experimental data.

Purification of Ins(1,4,5)P_3-specific antibodies

Protein A column chromatography (step 1) --- The antiserum (5.2 ml)

was applied to an Econo-Pac protein A cartridge (5 ml, Bio-Rad) equilibrated with 100 mM Tris/HCl, pH 8.0. The column was washed with, in sequence, 20 ml of the equilibrating buffer, and 25 ml of 10 mM Tris/HCl, pH 8.0. The bound IgG was eluted with 25 ml of 100 mM glycine buffer, pH 3.0, at a flow rate of 0.5 ml/min. Fractions of 1.7 ml were collected in tubes containing 100 μl of 1 M Tris/HCl, pH 8.0. Fractions 36-38, exhibiting anti-Ins(1,4,5)P_3 antibody activity were collected, concentrated by ultrafiltration, and dialyzed against 10 mM Tris/HCl, pH 7.5.

Ins(1,4,5)IP_3-agarose chromatography (step 2) --- The dialyzed solution from step 1 was applied to a Ins(1,4,5)IP_3-agarose column (5.8 ml bed volume) equilibrated with 10 mM Tris/HCl, pH 7.5. The column was washed with the equilibration buffer followed by 500 mM NaCl in the same buffer. The absorbed proteins were eluted with, in sequence, 50 ml of 100 mM glycine buffer, pH 3.5, 10 ml of 10 mM Tris/HCl, pH 8.8, and 50 ml of 100 mM $NaHCO_3/Na_2CO_3$ buffer, pH 10.5 Fractions of 1.7 ml were collected. For the eluate with the glycine buffer and with the $NaHCO_3/Na_2/CO_3$ buffer, fractions were collected in tubes containing 100 μl of 1 M Tris/HCl, pH 8.0, and 700 μl of 1 M Tris/HCl, pH 7.6, respectively. Fractions 66-69, exhibiting anti-Ins(1,4,5)IP_3 antibody activity, were pooled, concentrated, and dialyzed against 10 mM Tris/HCl, pH 7.5.

Results

Preparation of antigens

The design of the Ins(1,4,5)P_3 analogues, **1** and **2**, as haptens was based on: (a) the strategic importance of the C-2,3, *cis*-dihydroxyl groups, especially the axial 2-OH, in recognizing the microenvironments surrounding the phosphate functions, and (b) the potential steric effect of the linker on epitope recognition. The amine-bearing derivative **1** crosslinked to BSA using glutaraldehyde as a coupling agent, followed by in situ $NaBH_4$ reduction, to afford IP_3-C_6NC_5-BSA. On the other hand, the vicinal diol **2** was subjected to sodium periodate oxidation to yield the aldehyde intermediate, which,

without purification, was coupled to the amino functions of the carrier protein. In situ $NaBH_4$ reduction of the shiff base yielded IP_3-C_5-BSA. The phosphorous contents of IP_3-C_6-NC_5-BSA and IP_3-C_5-BSA, determined by elemental analysis, were 2.9% and 0.67%, respectively. Accordingly, the molar ratios of the bound Ins(1,4,5)P_3 to BSA were estimated to be 22 and 5, respectively.

Anti-Ins(1,4,5)P_3 antisera

Three New Zealand rabbits were immunized with IP_3-C_6NC_5-BSA, and a fourth one was injected with IP_3-C_5-BSA. The antibodies in the rabbit sera were detected by an ELISA where Ins(1,4,5)P_3 was covalently attached to the well surface through a C-6 linkage by reacting **1** with maleic anhydride-activated polystyrene plates. This antibody capture immunoassay provided a straightforward and consistent analysis of the antisera, and obviated tedious procedures associated with radioactive binding assays. Moreover, this assay avoided interference caused by nonspecific antibodies. For instance, an analysis using conventional EIA plates coated with Ins(1,4,5)P_3-C_6NC_5-casein was interfered by concomitant binding of the C_6NC_5-linker-directed antibodies to the exposed spacer on the carrier protein. As a consequence, this conventional assay did not respond to competitive inhibition by free Ins(1,4,5)P_3 in a quantitative manner.

After 2-3 times of booster injections, one of the rabbits immunized with IP_3-C_6NC_5-BSA and the rabbit receiving IP_3-C_5-BSA were found to produce antibodies against Ins(1,4,5)P_3, both with similar titers of about 1:4,000. This antibody formation seemed to be independent of spacer length and IP_3 content. The antibody titers increased only moderately in both rabbits after subsequent booster injections. It appeared that the remaining two rabbits which received IP_3-C_6NC_5-BSA immunization generated antibodies predominantly directed against the C_6NC_5-linker.

The avidity and specificity of these antisera were examined by competitive ELISA experiments between immobilized

$Ins(1,4,5)P_3$ and various inositol phosphates. As indicated from the concentrations at half-maximal absorbance (B/B_o = 0.5) or IC_{50} (Table 1), for the IP_3-C_6NC_5-BSA induced antiserum, the affinity toward various inositol phosphates was in the order of: $Ins(1,4,5)P_3$ > $Ins(1,3,4,5)P_4$ >> $Ins(1,3,4)P_3$ > $Ins(3,4,5,6)P_4$ > $Ins(1,5,6)P_3$, PIP_2, $Ins(1,2,5,6)P_4$ > $Ins(4,5)P_2$ > IP_6 > $Ins(1)P_1$.

Table 1

Competitor	[Competitor] (M) at half maximal absorbance (B/B_o = 0.5)	
	IP_3-C_6-NC_5-BSA induced antibodies	IP_3-C_5-BSA induced antibodies
(1,4,5)IP_3	8.9×10^{-8}	3.1×10^{-8}
(1,3,4,5)IP_4	2.0×10^{-7}	7.1×10^{-8}
(1,3,4)IP_3	1.7×10^{-6}	1.0×10^{-5}
(3,4,5,6)IP_4	4.5×10^{-6}	6.3×10^{-6}
PIP_2	1.6×10^{-5}	3.2×10^{-5}
(1,2,5,6)IP_4	2.1×10^{-5}	7.1×10^{-5}
(4,5)IP_2	3.8×10^{-5}	2.5×10^{-5}
IP_6	1.5×10^{-4}	8.0×10^{-4}
(1)IP_1	4.2×10^{-4}	N.D.

Table Legend

Table 1. **Avidity of antisera toward various inositol phosphates and PIP_2.** The avidity is expressed as the concentrations at half-maximal absorbance (B/B_o = 0.5) in the competitive ELISA experiments.

The recognition of inositol phosphates by the IP_3-C_5-BSA induced antiserum qualitatively paralleled that mentioned above (Table 1). But, the IP_3-C_5-BSA induced antibodies exhibited stronger binding toward both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 and a 10-fold decrease in affinity with Ins(1,3,4)P_3.

Although these antisera were highly specific for Ins(1,4,5)P_3, they also cross-reacted with Ins(1,3,4,5)P_4. The ratios of $IC_{50}^{[Ins(1,3,4,5)P_4]}$ to $IC_{50}^{[Ins(1,4,5)P_3]}$ were 2.2 and 2.3 for the antisera against IP_3-C_6NC_5-BSA and IP_3-C_5-BSA respectively.

Purification of Ins(1,4,5)P_3-specific antibodies

The level of discrimination between Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 by these antisera appeared to be lower than that reported for Ins(1,4,5)P_3 receptors, Guillemette, B.; Balla, T., Baukal, J.; and Catt, K.J. (1988) *J. Biol. Chem.* **263**, 4541-4548; Supattapone, S.; Worley, P.F.; Baraban, J.M.; and Snyder, S.H. (1988) *J. Biol. Chem.* **263**, 1530-1534; and Ferris, D.C.; Huganir, R.L.; Supattapone, S.; and Snyder, S.H. (1989) *Nature* **342**, 87-89. The lack of specificity could be attributed to the heterogeneity in the antigen binding sites. The IP_3-C_5-BSA induced antiserum was subjected to chromatographic purification on immobilized protein A and Ins(1,4,5)P_3-agarose. The utility of the IP_3 affinity column is especially noteworthy. The Ins(1,4,5)P_3-specific antibodies had strong binding with the affinity matrix, and could only be eluted under alkaline conditions.

Referring to Fig. 2, the IP_3-C_5-BSA induced antiserum (5.2 ml) was applied to an Econo-Pac protein A cartridge (5 ml, Bio-Rad) equilibrated with 100 mM Tris buffer, pH 8.0. The column was washed, in sequence, with (a) 20 ml of the equilibrating buffer, (b) 25 ml of 10 mM Tris buffer, pH 8.0, and (c) 25 ml of 100 mM glycine buffer, pH 3.0, at a flow rate of 0.5 ml/min. Fractions of 1.7 ml were collected Fractions 36-38, exhibiting anti-Ins(1,4,5)IP_3 antibody activity, were

pooled, concentrated and dialyzed against 10 mM Tris buffer, pH 7.5.

Referring to Fig. 2, the dialyzed solution was applied to a Ins(1,4,5)P_3-agarose column (5.8 ml bed volume) equilibrated with 10 mM Tris buffer, pH 7.5. The column was washed with equilibrating buffer followed by, in sequence, (a) 15 ml of 500 mM NaCl in the same buffer, (b) 30 ml of 100 mM glycine buffer, pH 3.5, (c) 15 ml of 10 mM Tris buffer, pH 8.8, and (d) 35 ml of 100 mM $NaHCO_3/Na_2CO_3$ buffer, pH 10.5. Fractions of 1.7 ml were collected. Fractions 66-69, exhibiting anti-Ins(1,4,5)P_3 antibody activity, were pooled, concentrated, and dialyzed against 10 mM Tris buffer, pH 7.5.

These affinity-purified antibodies showed much improved selectivity between Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4, with IC_{50} values of 12 nM and 730 nM, respectively. The differential affinity, as indicated by the ratios of IC_{50} values, increased from 2- to 60-fold.

Referring to Fig. 3, the displacement curves were generated from competitive ELISA experiments between immobilized (1,4,5)IP_3 and various inositol phosphates shown in the insert. The antibodies were preincubated with individual competitors at various concentrations for 30 minutes at room temperature before added to the (1,4,5)IP_3 immobilized EIA plates. The assay for the competitive binding was the same as that described for the ELISA under "Experimental Procedures". Percentage total specific binding is expressed by B/B_o, where B = absorbance with competitor and B_o = absorbance without competitor. Each data point represents the means of three determinations. For clarity, the standard deviations were not shown.

IC_{50} values for other inositol phosphates are: Ins(4,5)P_2, 14 μM; PIP_2, 14 μM; Ins(1,3,4)P_3, 39 μM; Ins(1,3,4,5,6)P_5, 45 μM, which are three orders or magnitude higher than that of Ins(1,4,5)P_3.

Nonspecific inhibition of Ins(1,4,5)P_3-antibody interactions by multivalent ions

In view of the electrostatic nature of Ins(1,4,5)P_3-

antibody recognition, the interaction might be interfered by anions that competitively bound to the positively charged active domain of the antibodies in a nonspecific manner. Fig. 5 shows that the binding between the affinity-purified antibodies and the immobilized Ins(1,4,5)P_3 was inhibited by a number of polyanionic substances at high concentrations. The IC_{50} values for individual inhibitors were: ATP^{4-}, 0.21 mM; HPO_4^{2-}, 2.61 mM; SO_4^{2-}, 11.6 mM.

Referring to Fig. 4, the affinity-purified antibodies were preincubated with individual multivalent ions at indicated concentrations for 30 minutes at room temperature before added to the (1,4,5)IP_3 immobilized EIA plates. The counterion for all the anions tested is Na+ which showed no inhibiting effect on the binding. The assay for the binding inhibition was the same as that described for the ELISA in the Experimental Procedures. Percentage maximal antibody binding is expressed by B/B_o, where B = absorbance with inhibitor and B_o = absorbance without inhibitor. Each data point represents the means of three determinations.

However, no appreciable interference was noted with haparin up to 0.3 mM, nor were with other monovalent anions such as N_3- and Cl-. Cations such as K+, Na+, NH_4+, and Tris at concentrations up to 100 mM did not cause significant inhibition to the antibody recognition.

Discussion

The invention is directed to the preparation of anti-Ins(1,4,5)P_3 antibodies through immunizing rabbits with covalent Ins(1,4,5)P_3-BSA conjugates. Both IP_3-C_6NC_5-BSA and IP_3-C_5-BSA were able to generate antibodies with a high degree of specificity for Ins(1,4,5)P_3. Both antiserum preparations showed discriminative binding toward Ins(1,4,5)P_3, but also cross-reacted with Ins(1,3,4,5)P_4 with one-third of the affinity. Other inositol phosphates including Ins(1)P_1, Ins(4,5)P_2, Ins(1,3,4)P_3, Ins(1,5,6)P_3, Ins(1,2,5,6)P_4, IP_6, Ins(3,4,5,6)P_4, and PIP_2 failed to effect sufficient molecular interactions with the antibodies, and thus exhibited much lower affinity by two to four orders of magnitude.

The problem in cross-reaction with Ins(1,3,4,5)P_4 could be overcome by affinity purification of the antisera on Ins(1,4,5)P_3-agarose. This affinity matrix is distinctly different from other types of Ins(1,4,5)P_3-based affinity absorbents reported in the literature, Prestwich, G.D.; Marecek, J.F.; Mourey, R.J.; Theibert, A.B.; Ferris, C.D.; Dannof, S.K.; and Snyder, S.H. (1991) *J. Am. Chem. Soc.* **113**, 1822-1825; and Tegge, W.; and Ballou, C.E. (1992) *Carbohydr. Res.* **230**, 63-77. The three phosphate functions of the immobilized Ins(1,4,5)P_3 are freely exposed, enabling the optimal interaction with the binding proteins. The degree of discrimination between Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 for the affinity-purified antibodies was comparable to that for Ins(1,4,5)P_3 receptors. Nahorski and his coworkers recently reported that Ins(1,3,4,5)P_4 was about 40-fold weaker than Ins(1,4,5)P_3 at displacing specific [^{3}H]Ins(1,4,5)P_3 binding from Ins(1,4,5)P_3 receptors, Wilcox, R.A.; Challiss, R.A.J.; Baudin, G.; Vasella, A.; Potter, B.V.L.; and Nohorski, S.R. (1993) *Biochem. J.* **294**, 191-194. The reported IC_{50} values for the tetrakis- and tris-phosphates were 762 nM and 20.7 nM, respectively, which are in line with those obtained with the purified antibodies. This cross interaction may be attributed to the largely shared structural motifs between these two polyphosphates.

The molecular basis of ligand recognition for these antibodies is analogous to that of Ins(1,4,5)P_3 receptors, arising from the complementary interactions involving ion pairing and hydrogen bonding between the binding domain and the hapten. The binding of the antibodies to immobilized Ins(1,4,5)P_3 could be disrupted by a number of unrelated multivalent anions including ATP^{4-}, HPO_4^{2-}, SO_4^{2-} at high concentrations, while no significant inhibition was noted with monovalent ions. However, unlike Ins(1,4,5)P_3 receptors, the interaction between the antibodies and the ligand was not affected by heparin up to 0.3 mM. These findings suggest that multivalent anions bound to and neutralized the charges of basic amino acid residues inside the IP_3 binding domain. This

inhibition is noteworthy because use of any of these anions during the antibody preparation and immunoassay will lead to false negative results. In our study, attempts to enrich the antibodies using $(NH_4)_2SO_4$ precipitation resulted in a complete loss of binding capability even after extensive dialysis. In the literature, inhibition of binding by multivalent anions has also been reported for Ins(1,4,5)P_3 receptors. The antagonistic action of heparin in Ins(1,4,5)P_3-induced Ca^{2+} mobilization is well understood, Hill, T.D.; Berggren, P.-O.; Boynton, A.L. (1987) *Biochem. Biophys. Res. Commun.* **149**, 897-901; Guillemette, G.; Lamontagne, S.; Boulay, B.; and Mouillac, B. (1989) *Mol. Pharmacol.* **35**, 339-344; Joseph. S.K.; and Rice, h.L. (1989) *Mol. Pharmacol.* **35**, 355-359; and Ghosh, T.K., Eis, P.S.; Mullaney, J.M.; Ebert, C.L.; and Gill, D.L. (1988) *J. Biol. Chem.* **362**, 11075-11079. Also, nucleotides and phosphate have been reported to inhibit the binding of Ins(1,4,5)P_3 to the receptor from the cerebellar membrane, Willcocks, A.L.; Cooke, A.M.; Potter, B.V.L.; and Nohorski, S.K. (1987) *Biochem. Biophys. Res. Commun.* **146**, 1071-1078; and Maeda, N.; Kawasaki, T.; Nakade, S.; Nobutaka, Y.; Takahisa, T.; Kasai, M.; and Mikoshiba, K. (1991) *J. Biol. Chem.* **266**, 1109-1116. Evidently, the inhibition of Ins(1,4,5)P_3 binding by various multivalent anions further underscores the analogy of the antibody binding to the receptor recognition. On the other hand, cations such as K+, Na+, etc. did not affect the binding.

A technical note worth mentioning is the ELISA developed in this study. In our initial experiments, IP_3-C_6NC_5-casein, prepared in the same manner as that described for our BSA conjugate, was coated to regular microtiter plates through adsorption. These plates failed to bind selectively with IP_3 antibodies, and did not respond to competitive binding experiments by free IP_3 in a concentration-dependent manner. This lack of specific binding may be attributed to (a) the presence of antibodies directed against C_6NC_5-linker, or (b) the lack of adsorption of IP_3-C_6NC_5-casein to polystyrene surface due to high charge density. Consequently, with the IP_3 molecules covalently attached to the microtiter plates as

described, these potential interferences were circumvented.

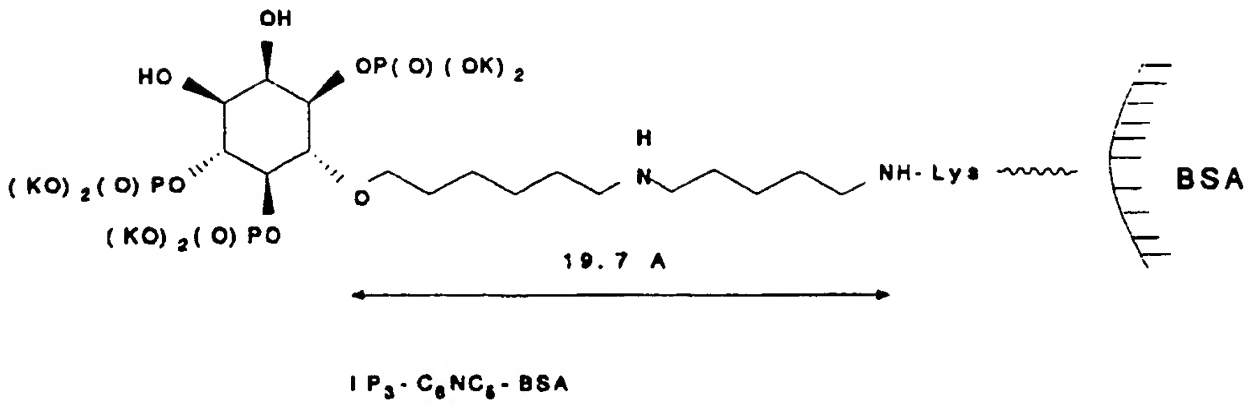
The utility of anti-Ins(1,4,5)P_3 antibodies is 3-fold. First, the ELISA developed here offers an easy quantitative analysis of Ins(1,4,5)P_3. Second, antibodies directed against phosphatidylinositol and PIP_2 have been applied to probing the intracellular transduction mechanism in various types of cells involving PIP_2 as a second messenger precursor, Matuoka, K.; Fukami, K.; Nakanishi, O.; Kawai, S.; and Takenawa, T. (1988) *Science* **239**, 640-643; Fukami, K.; Matsuoka, K.; Nakanishi, O.; Yamakawa, A.; Kawai, S.; and Takenawa, T. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 9057-9061; Huang, C.-L.; Takenawa, T.; and Ives, H.E. (1991) *J. Biol. Chem.* **266**, 4045-4048; and Loirand, G.; Faiderbe, S.; Baron, A.; Geffard, M.; and Mironneau, J. (1992) *J. Biol. Chem.* **267**, 4312-4316. These anti-Ins(1,4,5)P_3 antibodies developed by us now add a new line of biological probes for examining the actions of Ins(1,4,5)P_3 and its metabolites. Third, the recent development of the anti-idiotypic mimicry of biological ligands has become a useful tool in studying receptor functions, Gaulton, G.N.; and Greene, M.I. (1986) *Ann. Rev. Immunol.* **4**, 253-280; and Linthicum, D.S.; and Farid, N. (eds) (1988) in *Anti-idiotypes, Receptors and Molecular Mimicry*, Spring-Verlag, New York; pp. 1-322. Certain anti-idiotypic antibodies have been demonstrated to mimic biological activities of endogenous ligands by acting as internal images. Examples include anti-idiotypic antibodies against glutamate, Duce, I.R.; Budd, T.C.; and Richardson, P.J. (1991) *Biochem. Soc. Trans.* **19**, 143-146, dopamine, Mons. N.; Dubourg, P.; Messier, C.; Chiavaroli, C.; Calas, A.; and Geffard, M. (1991) *J. Hirnforsch.* **32**, 617-625, substance P, Couraud, J.Y.; Maillet, S.; Grassi, J.; Frobert, Y.; and Pradelles, P. (1989) *Methods Enzymol.* **178**, 275-300, and platelet activating factor, Wang, C.-J.; and Tai, H.-H. (1991) *J. Biol. Chem.* **266**, 12372-12378.

The foregoing description has been limited to a specific embodiment of the invention. It will be apparent, however, that variations and modifications can be made to the

invention, with the attainment of some or all of the advantages of the invention. Therefore, it is the object of the appended claims to cover all such variations and modifications as come within the true spirit and scope of the invention.

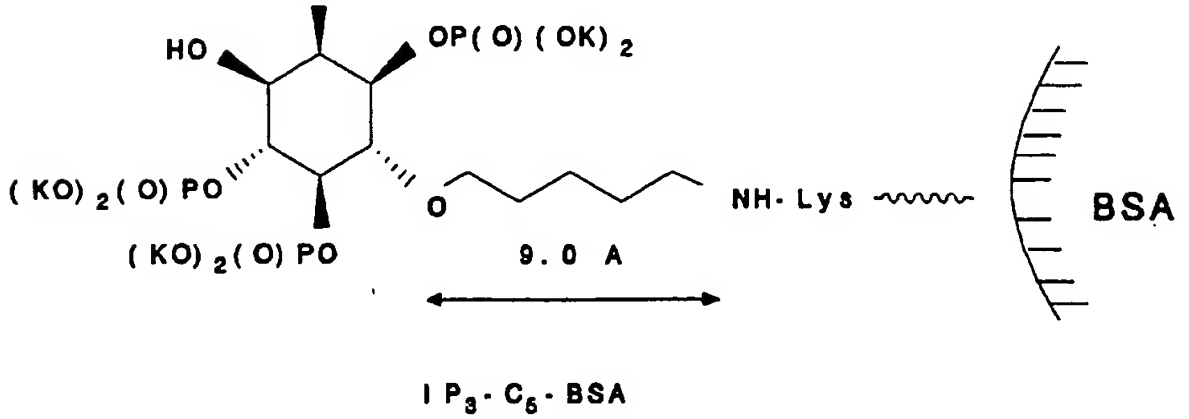
Having described the invention, what we now claim is:

1 1. An antigenic compound having the structure:



I P_3- C_6NC_6- BSA

1 2. An antigenic compound having the structure of:



I P_3- C_6- BSA

1 3. An Ins(1,4,5)P_3 polyclonal antibody which binds to
2 the antigenetic compound of claims 1 or 2.

1 4. The polyclonal antibodies of claim 3 wherein the
2 Ins(1,4,5)P_3 antibodies are produced through immunization of
3 a host vertebrate with covalent Ins(1,4,5)P_3-BSA conjugates.

1 5. The polyclonal antibody of claim 3 which is of an

IgG isotype.

6. The polyclonal antibody of claim 3 characterized in that the binding is the result of ion pairing and hydrogen bonding between the binding domain of the antibody and the hapten of either compound of claims 1 or 2.

7. An ELISA for the detection of antibodies responsive to the compounds of claims 1 or 2 which comprises:

providing a maleic anhydride-activated polystyrene surface;

attaching either compound 1 or 2 to the activated surface via a C-6 linkage to form an affinity matrix; and

binding the Ins(1,4,5)P_3 antibodies to the affinity matrix.

8. A method of producing a polyclonal antibody of claim 4 comprising:

immunizing a vertebrate host with an immunogenic composition of claims 1 or 2;

removing antisera from the vertebrate host;

contacting an affinity matrix comprising either compound 1 or 2 with the antisera;

binding the antibodies to the hapten of either compound 1 or 2; and

detecting the presence of the Ins(1,4,5)P_3 antibody.

9. The method of claim 8 which comprises:

isolating the antibody; and

recovering the antibody.

10. The method of claim 8 which comprises assessing the antibody binding by adding goat anti-rabbit IgG-hrp conjugate; and

measuring the absorbance to determine the degree of binding.

11. A method for the preparation of an IP_3-C_6NC_5-BSA

conjugate which comprises:

preparing an aqueous solution of IP_3-C_6NC_5-BSA; and

lyphosizing said solution to provide the IP_3-C_6-NC_5-BSA.

12. A method for the preparation of an IP_3-C_5-BSA conjugate which comprises:

preparing an aqueous solution of IP_3-C_5-BSA;

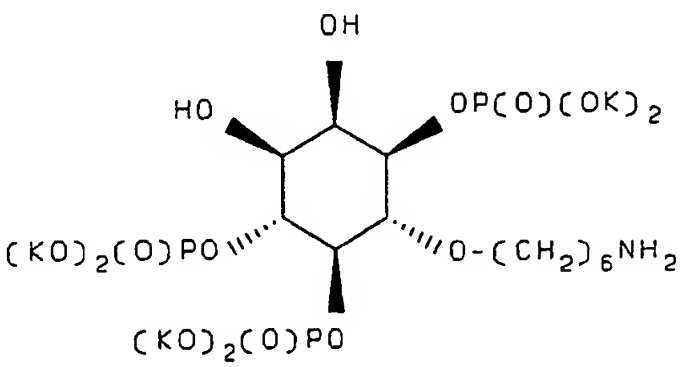
lyphosizing said solution to provide 6-0-(4'-formylbutyl)-D-myo-inositol 1,4,5 triphosphate;

lyphosizing said 6-0-(4'-formylbutyl)-D-myo-inositol 1,4,5 triphosphate to provide IP_3-C_5-BSA.

13. An affinity matrix for an ELISA assay which comprises:

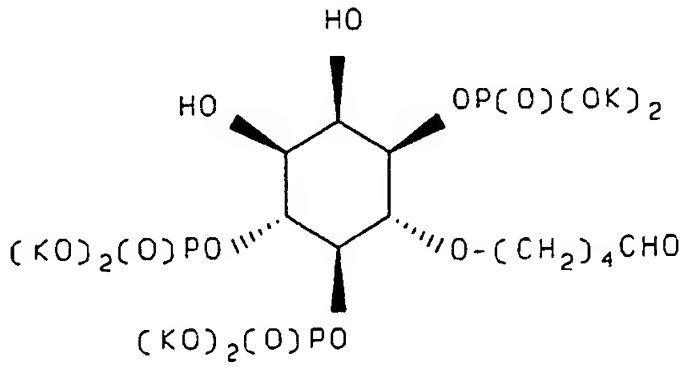
a maleic anhydride-activated polystyrene having either compound one or two attached thereto.

14. A compound of the structural formula:



(-)-1

15. A compound of the structural formula:



(-)-2

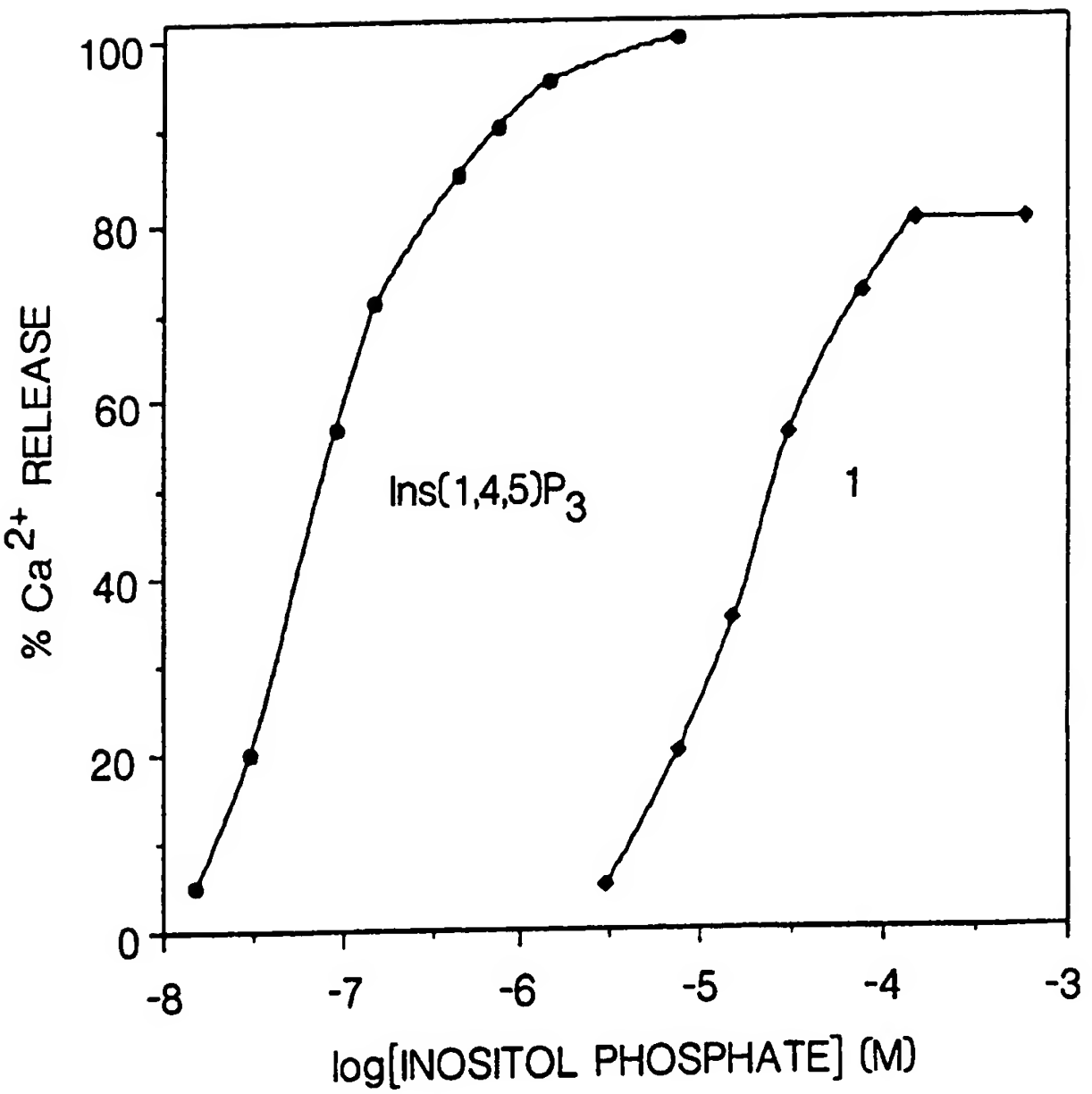

100
80
60
40
20
0
% Ca^{2+} RELEASE
Ins(1,4,5)P_3
1
-8
-7
-6
-5
-4
-3
log[INOSITOL PHOSPHATE] (M)

FIG. 1

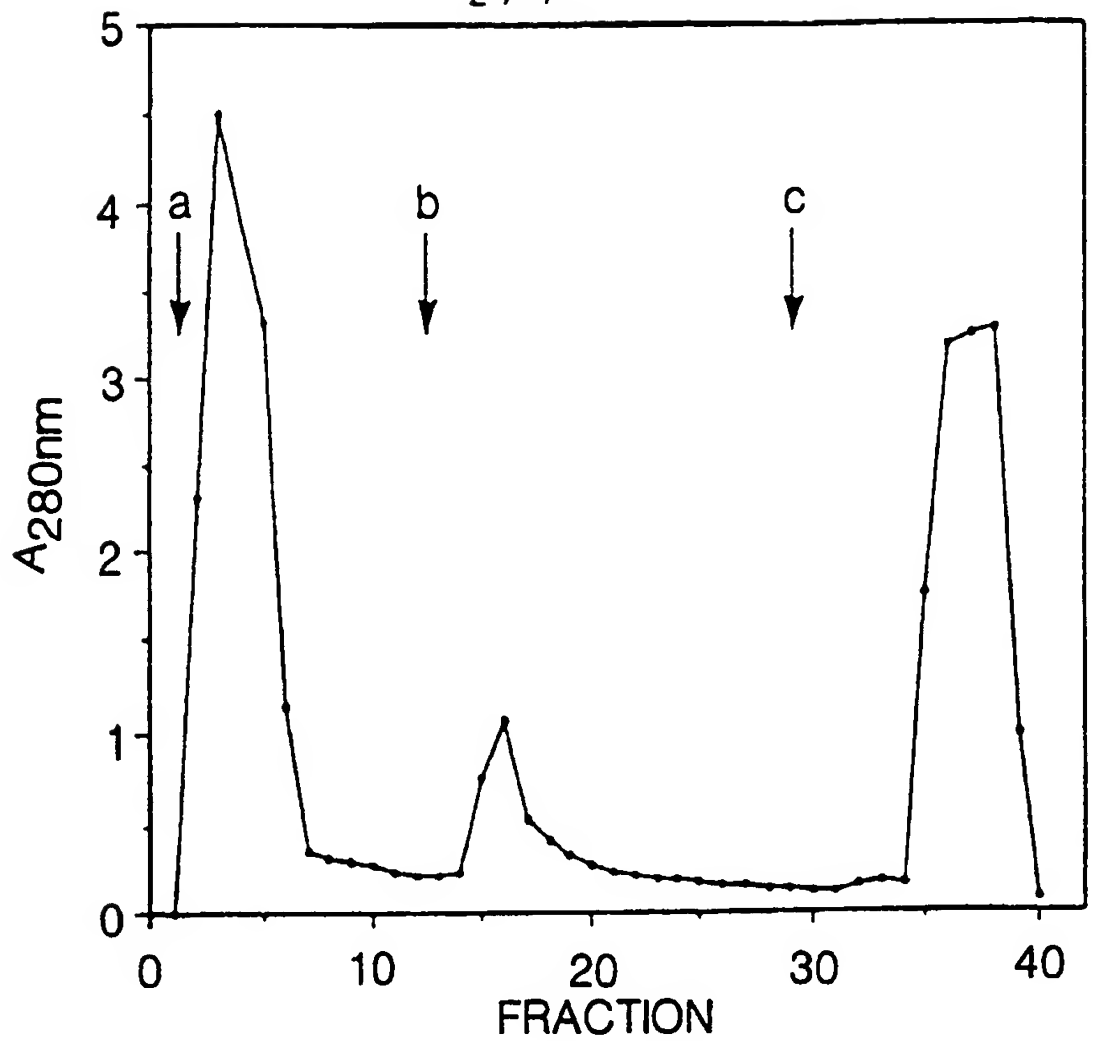

5
4
3
2
1
0
A_{280nm}
a
b
c
0
10
20
30
40
FRACTION

FIG. 2A

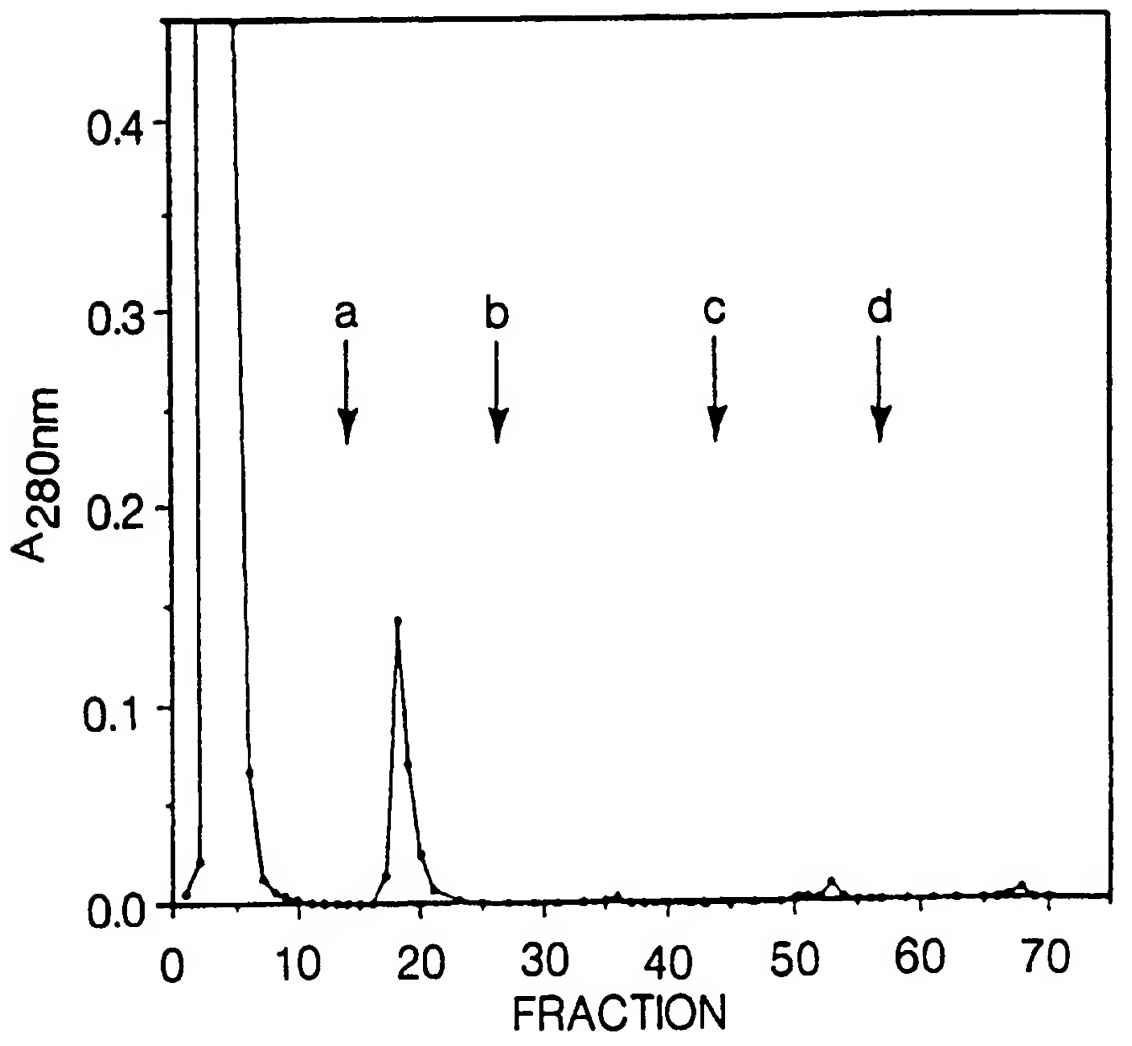

0.4
0.3
0.2
0.1
0.0
A_{280nm}
a
b
c
d
0
10
20
30
40
50
60
70
FRACTION

FIG. 2B

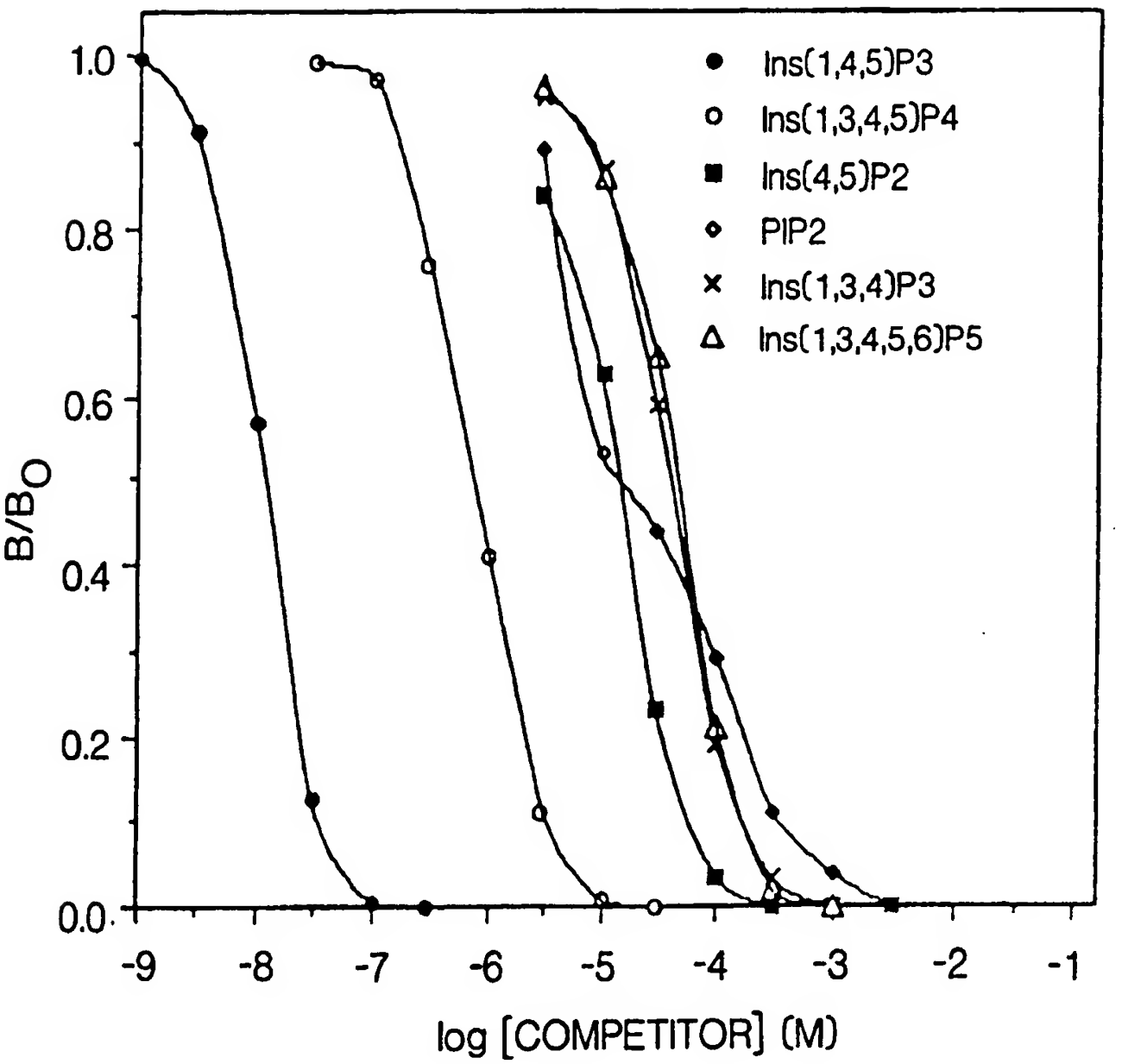

1.0
0.8
0.6
0.4
0.2
0.0
B/B$_O$
-9
-8
-7
-6
-5
-4
-3
-2
-1
log [COMPETITOR] (M)
Ins(1,4,5)P3
Ins(1,3,4,5)P4
Ins(4,5)P2
PIP2
Ins(1,3,4)P3
Ins(1,3,4,5,6)P5

FIG. 3

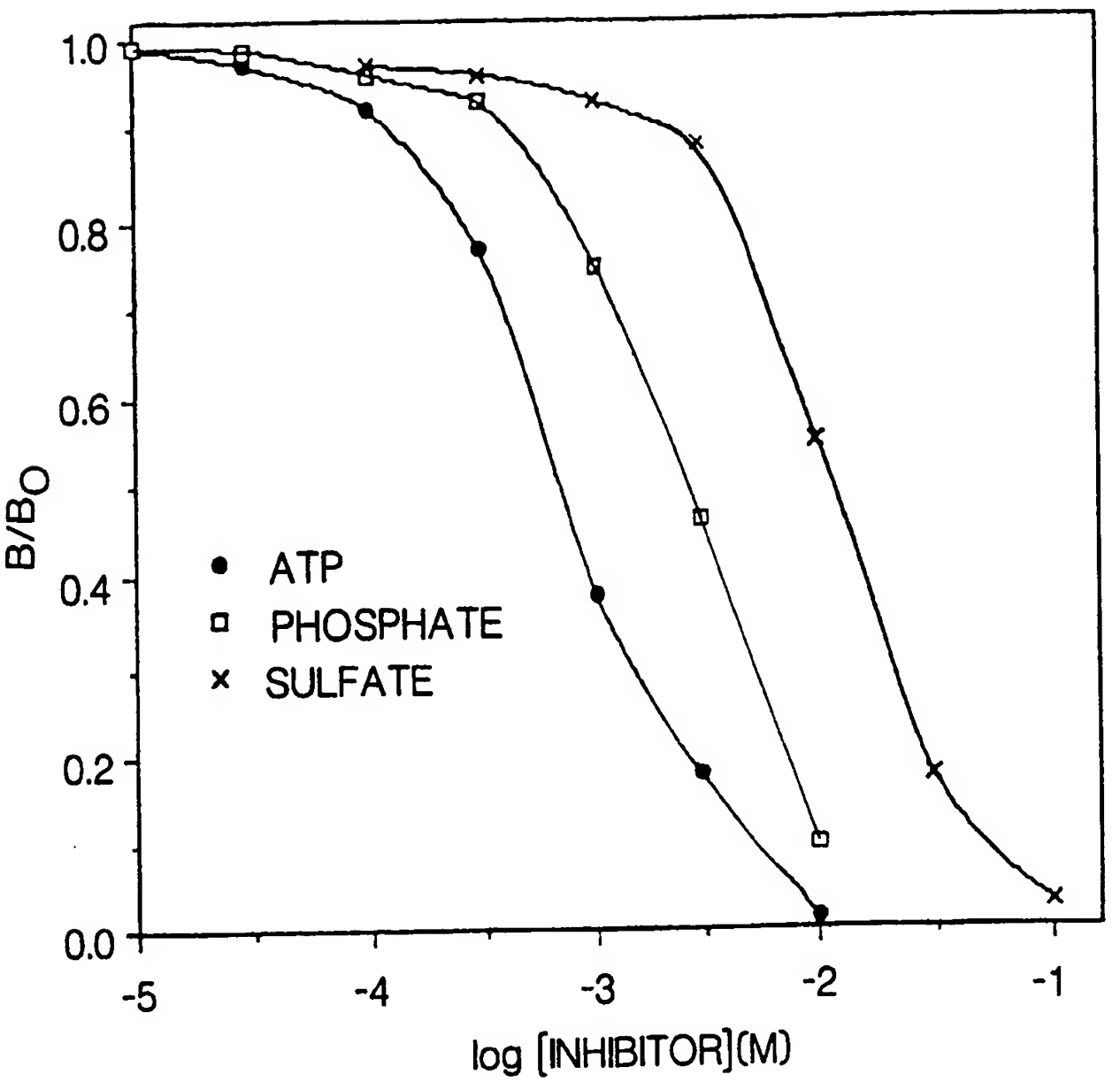

1.0
0.8
0.6
0.4
0.2
0.0
B/B$_O$
ATP
PHOSPHATE
SULFATE
-5
-4
-3
-2
-1
log [INHIBITOR](M)

FIG. 4

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07K 14/47, 17/00; C07F 9/117

US CL : 530/363, 405; 558/161

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/363, 405; 558/161

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 4,873,355 (HOBBS ET AL.) 10 October 1989, the entire document.	1, 7, 11, 13
A	US, A, 5,210,263 (KOZIKOWSKI ET AL.) 11 May 1993, the entire document.	1, 7, 11, 13
A	US, A, 5,260,472 (CHEN) 09 November 1993, the entire document.	1, 7, 11, 13
A,P	US, A, 5,292,913 (OZAKI ET AL.) 08 March 1994, the entire document.	1, 7, 11, 13
Y	US, A, 5,252,707 (OZAKI ET AL.) 12 October 1993, see col. 5, line 54 to col. 6, line 26, in particular.	11

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

27 FEBRUARY 1995

Date of mailing of the international search report

06 APR 1995

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
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Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,347,176 (MEHTA) 31 August 1982, see col. 2. line 43 and col. 17, lines 11-17, in particular.	11

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1, 7, 11 and 13

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

International application No.
PCT/US95/00412

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1, 7, 11 and 13, drawn to an antigenic compound, a method, a process and an affinity matrix.

Group II, claim(s) 2 and 12, drawn to an antigenic compound and a process.

Group III, claim(s) 3-6 and 8-10, drawn to an antibody and a process.

Group IV, claim 14, drawn to a compound.

Group V, claim 15, drawn to a compound.

The inventions listed as Groups I-V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The inventions as grouped are distinct, each from the other, because they represent different inventive endeavors. The antigenic compound in Group I would not suggest the antigenic compound in Group II, the antibody in Group III, the compound in Group IV or the compound in Group V. Groups I-V are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.